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Efficacy of *Lawsonia inermis* leaves extract and its phenolic compounds against olive knot and crown gall diseases

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ABSTRACT

This study was undertaken to determine the antibacterial efficacy of the leaves extracts of *Lawsonia inermis in vitro* and *in vivo*, against the phytopathogenic bacteria *Pseudomonas savastanoi* pv *savastanoi* IVIA 1628 and *Agrobacterium tumefaciens*. The hydroalcoholic extract of *L. inermis* was fractionated by liquid–liquid partition using hexane, chloroform, ethyl acetate and butanol. Among the tested fractions, the ethyl acetate (EtOAcF) and chloroform ($CHCl_3F$) extracts exhibited high inhibition against all tested plant pathogens. The extracts exerted a bactericidal activity against both *P. savastanoi* and *A. tumefaciens* with MIC values ranging from 1.25 to 2.5 mg/ml. *In planta* experiments, the EtOAcF, tested at four concentrations (0.4, 0.2, 0.1 and 0.05 mg/wounds), completely inhibited the formation of knots on twigs of olive and tomato plants inoculated with pathogenic strains of *P. savastanoi* and *A. tumefaciens* (strains B6 and C58). The phytochemical screening revealed that the *L. inermis* fractions contain flavonoids, quinones, tannins and terpenoids. The qualitative analysis of the EtOAcF by LC–DAD–ELSD–ESI/MS^n showed the presence of eight identified phenolic and phenolic glycoside compounds. These results suggest that *L. inermis* extracts could be used to control plant bacterial diseases caused by *P. savastanoi* and *A. tumefaciens*.

1. Introduction

Plants are constantly exposed to environmental stresses and threatened by a variety of pathogenic microorganisms present in their environments. Bacterial diseases caused by specifically *Pseudomonas savastanoi* pv. *savastanoi*, a pathogen to plants of the Oleaceae family, and *Agrobacterium tumefaciens*, the causal agent of crown gall diseases on various host plants, lead to considerable losses in productivity and quality of harvests (Escobar and Dandekar, 2003; Hammami et al., 2009). For many years, a variety of different chemical and synthetic compounds have been used as antimicrobial agents to inhibit the plant pathogenic bacteria. However, these chemical pesticides usually take a long time to be degraded completely, which may cause heavy toxicity to human and domestic animals (Damalas and Eleftherohorinos, 2011). In addition, plant pathogens are prone to develop 'drug' resistances leading to a decrease in the effectiveness of chemical pesticides (Rosenberger and Meyer, 1981). Consequently, there is an obvious need to develop biopesticides products for disease control, especially biopesticides endowed with activities against plant pathogenic bacteria that acquired resistance to commercial pesticides (Franco et al., 2007).

Research focused on plant-derived natural bactericides and their possible applications in agriculture to control plant bacterial diseases is being intensified. Plant secondary metabolites have enormous potential to inspire and influence modern agrochemical research. Essential oils and organic extracts could be a source of alternative classes of natural compounds in controlling plant pathogens. They have been suggested as effective substitutes for synthesised chemical pesticides (Gan-Mor and Matthews, 2003).

Lawsonia inermis (Lythraceae), commonly called "henna" in North Africa, is widely used in traditional medicine to treat various diseases and well known worldwide for cosmetic use. The used *L. inermis* parts are bark, leaves, flowers and seeds. The leaves of *L. inermis* have been reported to contain various compounds like coumarins, flavonoids, gallic acid, naphthoquinones, naphthalene

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derivatives, triterpenoids, phenolic glycosides and xanthones (Chaudhary et al., 2010; Hsouna et al., 2011). *L. inermis* exhibits a variety of biological activities such as antimicrobial activity (El-Hag et al., 2007), hepatoprotective activity (Anaad et al., 1992), cytotoxic activity (Ali and Grever, 1998), anti-inflammatory, antipyretic and analgesic activities (Ali et al., 1995). However, to the best of our knowledge, there is no detailed report available in the literature on the *in vitro* antibacterial activity of organic extracts of *L. inermis* against plant pathogenic bacteria. Hence, the objectives of the present study were to investigate, (i) the antibacterial activity of *n*-Hexane, chloroform, ethyl acetate, *n*-butanol and water extracts of *L. inermis* leaves against plant pathogenic bacteria, (ii) verify the antibacterial effect of the active extract against *P. savastanoi* and *A. tumefaciens* in pot experiment in order to control symptom expression of olive knot and crown gall disease and (iii) determine the chemical composition of the most active extract.

2. Materials and methods

2.1. Plant material

L. inermis leaves were collected from Sfax (Tunisia, 35°14′58.36″ N, +11°7′17.75″ E). After the botanical identification of the species, a voucher specimen (LBPes L.W. 16.04) was deposited in the herbarium of the Biopesticides Team (LPIP) of the Center of Biotechnology of Sfax.

2.2. Preparation of plant extracts

The air-dried *L. inermis* leaves were ground to a fine powder using a grinder and the resulted material (100 g) was extracted by maceration into 600 ml of aqueous ethanol (ethanol/water, 4:1, v/v) at room temperature with occasional shaking. After three days, the extract was filtered (45 μm) and was concentrated under vacuum. The resulted residue (22 g) was suspended in 100 ml of distilled water and sequentially partitioned into *n*-hexane (3 × 250 ml), chloroform (3 × 250 ml), ethyl acetate (3 × 250 ml) and *n*-butanol (3 × 250 ml). The resulted five solutions were concentrated in a vacuum to obtain *n*-hexane (*n*-HexF; 1.92 g), chloroform ($CHCl_3F$; 0.28 g), ethyl acetate (EtOAcF; 1.97 g), *n*-butanol (*n*-BuOHF; 6.41 g) and water (WF; 8.84 g) fractions. These fractions were kept at 4 °C in the dark until further analysis.

2.3. Phytochemical tests

Biochemical tests for major *L. inermis* fractions were performed according to Allen (1974) and Harborne (1984). The tests were based on the visual observation of colour change or formation of a precipitate after the addition of specific reagents. The different chemical constituents tested include alkaloids, flavonoids, quinones, tannins and terpenoids.

2.4. In vitro antibacterial effect of L. inermis against plant pathogenic bacteria

2.4.1. Microorganisms

Five plant pathogenic bacteria were tested: strains B6, C58, ATCC 4720, and AR 125 of *A. tumefaciens* and *P. savastanoi* pv *savastanoi* IVIA 1628. These strains were kindly provided by the Olive Institute of Sfax – Tunisia. Agrobacterium strains were cultivated in Mannitol Glutamate Agar (MGA) containing in g/l (5 D mannitol, 2 L-glutamic acid, 0.5 KH_2PO4, 0.2 $MgSO_4$, $7H_2O$, agar, 20; pH 7.2). *P. savastanoi* was cultured in King B medium (KB) containing in g/l (20 peptone, 1,5 $MgSO_4$; 1,2 K_2HPO_4 and 15 ml glycerol) at 30 °C for 48 h. Inocula were prepared by adjusting the turbidity of each bacterial culture to 10^8 colony forming units/ml (cfu/ml).

2.4.2. Antibacterial effect of L. inermis against plant pathogenic bacteria

Antimicrobial activities of the *L. inermis* extracts were evaluated by means of agar-well diffusion assay according to the method of Andrews (2005) with minor modifications. Fifteen millilitres of the molten agar (45 °C) were poured into sterile petri dishes (Ø 90 mm). Cell suspensions were prepared and 100 μl was inoculated onto the surface of agar plates. Thereafter, wells with 6 mm in diameter were punched in the inoculated agar medium with sterilized Pasteur pipettes and the extracts were added to each well. Negative controls consisting of 20% DMSO and 50% ethanol were used to dissolve the plant extracts. The plate was allowed to stand for 2 h at 4 °C to permit the diffusion of the extracts followed by incubation at 30 °C for 48 h. The antibacterial activity was evaluated by measuring the zones of inhibition (clear zone around the well) against the tested microorganisms. All tests were repeated three times.

2.4.3. Determination of the minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC)

Minimum inhibitory concentrations (MICs) of *L. inermis* were determined according to Eloff (1998) in sterile 96-well microplates with a final volume of 100 μl in each microplate well. A stock solution of the extract (50 mg/ml) was prepared in 20% DMSO or in 50% ethanol. Thereafter, a two-fold serial dilution of the extract was prepared in the microplate wells over the range 0.039–10 mg/ml. To each test well, 5 μl of cell suspension was added to reach a final inoculum concentration of 10^6 cfu/ml. Negative controls consisting of 20% DMSO and 50% ethanol were used to dissolve the plant extracts. The plates were then covered with sterile plate covers and incubated at 30 °C for 48 h. The MIC was defined as the lowest concentration of the extract at which the microorganism does not demonstrate visible growth after incubation. As an indicator of microorganism growth, 25 μl of *p*-iodonitrotetrazolium violet (INT) at a concentration of 0.5 mg/ml, dissolved in sterile water, were added to the wells and incubated at 30 °C for 30 min. The formation of red chromogen indicates the occurrence of microorganisms. The MIC value was determined as the lowest concentration of the test sample in the well that did not form red colour.

For MBC determination, 10 μl liquid from each well that showed no colour change was plated on nutrient agar and incubated at 30 °C for 48 h. The lowest concentration that yielded no growth after this sub-culturing was taken as the MBC, indicating that >99.9% of the original inoculum was killed. The determination of MIC and MBC values was properly replicated three times and a fourth test was performed if the values were not identical.

2.5. In planta experiments

2.5.1. Suppression of crown gall disease in pot experiments

One-month-old tomato plants (cv. Riograndy) were used for *in vivo* tests. They were grown in a greenhouse in 15-cm-diameter pots containing a sterilized mix of soil–sand–peat (2:1:1 by volume) and watered daily by drip-irrigation. A mineral solution (NPK 20–20–20 + B + Cu + Fe + Mn + Mo + Zn 1–5–30–10–10–10, respectively) at 2 g/1 was distributed weekly into the pots to maintain optimum nutritional conditions. Temperature was maintained at 25 ± 2 °C and 15 ± 2 °C, respectively at day and night temperatures. The relative humidity was maintained between 70 and 80%, during the whole experiment, by using automatic cooling.